Suprabasal Induction of Ornithine Decarboxylase in Adult Mouse Skin Is Sufficient to Activate Keratinocytes

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To study the effects of *de novo* induction of ornithine decarboxylase (ODC) activity in adult, quiescent skin, we generated transgenic mice in which the suprabasal expression of an inducible form of the ODC protein fused to a modified estrogen receptor ligand-binding domain (ODCER) is driven by an involucrin promoter. After topical treatment with the inducing agent 4-hydroxytamoxifen (4OHT), ODC activity and putrescine levels were dramatically increased in the epidermis but not in the dermis of transgenic mice. 4OHT treatment stimulated both proliferation as measured by bromodeoxyuridine incorporation in basal epidermal cells and differentiation shown by increased expression of differentiation markers. Furthermore, induction of ODC activity did not rescue primary epidermal keratinocyte cultures isolated from ODCER2 mice from a calcium-triggered DNA synthesis block, as measured by [^{3}H]thymidine incorporation. *In vivo* induction of epidermal ODC enzyme activity significantly stimulated the vascularization of ODCER transgenic skin. Increased expression of interleukin-1β and keratin 6, markers of keratinocyte activation seen in wound healing, was also observed in 4OHT-treated transgenic skin. These results suggest that *de novo* suprabasal induction of ODC activity in adult mouse skin activates keratinocytes and stimulates vascularization in the dermal layer in a manner similar to skin undergoing wound healing.

Key words: angiogenesis/ornithine decarboxylase/polyamines/proliferation/skin carcinogenesis
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Polyamines have long been known to be associated with cell proliferation in both normal and neoplastic tissues (Tabor and Tabor, 1984; Pegg, 1988). Ornithine decarboxylase (ODC) is the initial rate-limiting enzyme involved in polyamine biosynthesis and is responsible for converting L-ornithine to putrescine. The polyamines spermidine and spermine are the most cationic small molecules of the cell, and the majority of cellular polyamines are bound to polyanionic macromolecules such as DNA, RNA, and phospholipids (Igarashi *et al*, 1982; Davis *et al*, 1992). Elevated levels of ODC and increased polyamines are purported to play an important role in tumorigenesis largely because of the early induction of ODC by tumor promoters (O'Brien, 1976; Gilmour *et al*, 1986, 1987) and to studies using inhibitors of ODC (Bollag, 1972; Verma *et al*, 1980; Weeks *et al*, 1982; Takigawa *et al*, 1983). For instance, α-difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ODC enzyme activity, inhibits the development of skin tumors in carcinogen-treated mice when it is given during the promotion phase (Weeks *et al*, 1982; Takigawa *et al*, 1983).

Previous studies using the keratin 6 (K6)/ODC transgenic mouse have shown that elevated levels of ODC directed to the outer root sheath cells of hair follicles using a K6 promoter over the lifetime of the animal lead to hair follicle degeneration and the subsequent formation of follicular cysts in the dermis (Megosh *et al*, 1995). The epithelial cells lining the follicular cysts express high levels of ODC and demonstrate a high proliferative index (Gilmour *et al*, 1999). By crossing the K6/ODC transgenic mouse with the Tg.AC v-Ha-*ras* transgenic mouse, we have demonstrated that elevated levels of ODC and polyamines cooperate with a mutated Ha-*ras* gene to promote epithelial tumor formation and invasion in mice (Smith *et al*, 1998). It is not known, however, as to how the abnormal skin phenotype of the K6/ODC transgenic mouse may contribute to changes in proliferation and tumorigenesis. For instance, the K6/ODC transgene is expressed throughout most of the development and lifetime of the mouse. In order to study the effects of elevated levels of ODC enzyme activity and polyamines on epidermal cells residing in normal adult skin with no hair loss, we have generated a transgenic mouse model in which an inducible form of ODC is targeted to suprabasal epidermal cells by an involucrin promoter. Suprabasal cells in intact, normal adult skin are no longer proliferating and are committed to differentiate terminally. Normally ODC is expressed, in basal proliferating cells in the epidermis. Cycling cells that express high levels of ODC, however, can be found also in suprabasal layers of skin tumors. Using transgenic mice with inducible epidermal ODC, we sought to determine whether forced ODC induction in suprabasal cells in the interfollicular epidermis of adult mice is sufficient to activate these cells. We show that *de novo* induction of ODC enzyme activity in adult mouse skin stimulates kera-

Abbreviations: BrdU, bromodeoxyuridine; DFMO, α-difluoromethylornithine; ER, estrogen receptor; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSP90, heat shock protein 90; IL, interleukin; K6, keratin 6; ODC, ornithine decarboxylase; 4OHT, 4-hydroxytamoxifen; α-SMA, α-smooth muscle actin

tinocytes and dermal stromal cells in a manner similar to that in wounded skin with increased epithelial proliferation and vascularization.

Results

Generation of ODCER transgenic mice To generate a transgenic mouse model that inducibly elevates ODC activity in the epidermis, a cDNA encoding a truncated murine ODC protein (Ghoda *et al*, 1989; Clifford *et al*, 1995) was fused in frame to a 4-hydroxytamoxifen (4OHT)-responsive mutant estrogen receptor (ER) ligand-binding domain (Littlewood *et al*, 1995) and then cloned downstream of the human involucrin promoter (Carroll *et al*, 1993). Following pronuclear microinjection, three independent founder lines were generated, all of which expressed the 85 kDa ODCER fusion protein in the epidermis (Fig 1*A*).

All of the ODCER transgenic mice were viable and fertile. Whereas the ODCER2 line exhibited no detectable phenotype, the ODCER1 and ODCER3 transgenic lines eventually lost their hair and developed follicular cysts in the dermis by 2 mo of age in the absence of 4OHT treatment (Fig 2*A*, *B*). The alopecia found in the ODCER1 and ODCER3 transgenic lines was accompanied by higher basal ODC enzyme activity in their skin compared with that in skin from ODCER2 transgenic mice or normal littermates (Fig 1*B*). ODCER2 transgenic mice were further characterized since they demonstrated a low basal ODC activity similar to wild-type mice and a normal skin phenotype with no hair loss or development of dermal cysts (Fig 2*C*) as seen in the ODCER1 and ODCER3 lines.

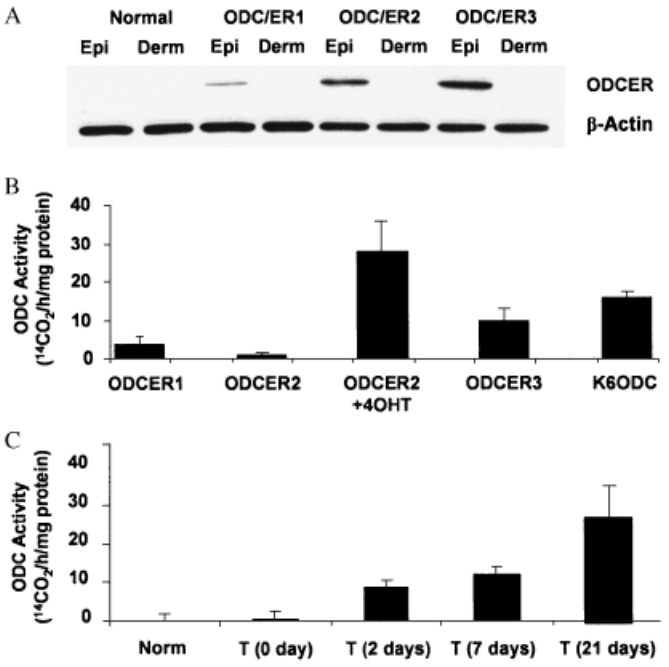

Figure 1
Induction of ornithine decarboxylase (ODC) enzyme activity in ODCER2 transgenic mice. (*A*) RIPA lysates were prepared from epidermal (Epi) and dermal (Derm) tissue from non-treated ODCER1, ODCER2, and ODCER3 transgenic mice and their non-transgenic littermates (normal). Equal amounts of protein were loaded and resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. ODCER and β-actin protein were detected by immunoblotting. A protein band between 80 and 90 kDa (corresponding to the ODCER fusion protein) was detected in transgenic epidermis but not in normal mouse skin using an antibody specific for mouse estrogen receptor (ER) protein. (*B*) ODC activity of total skin from ODCER1-3 transgenic mice, ODCER2 mice topically treated for 21 d with 4-hydroxytamoxifen (4OHT), and keratin 6 (K6)/ODC transgenic mice. Skin tissue from three different founder lines ODCER transgenic mice and their normal littermates as well as from K6/ODC transgenic mice was homogenized and assayed for ODC enzyme activity by quantifying the production of $^{14}CO_2$ from L-[^{14}C]ornithine. (*C*) Induction of ODC enzyme activity in total skin of 4OHT-treated ODCER2 transgenic mice. ODC/ER2 mice were topically treated with 1.0 mg 4OHT in 100 μL ethanol daily for 2, 7, and 21 d. Total skin was homogenized and assayed for ODC enzyme activity. ODC enzyme activity is expressed as nmol $^{14}CO_2$ per h per mg protein ± SD with at least three mice per treatment group.

Activation of ODCER in suprabasal transgenic epidermis We tested whether topical application of 4OHT would induce ODC enzyme activity in the skin of ODCER2 mice. Following daily applications of 4OHT (1 mg in 0.1 mL ethanol) for up to 21 d, ODC enzyme activity continued to increase to 8.7 and to 23.4 nmol $^{14}CO_2$ per h per mg protein after 2 and 21 d of treatment, respectively (Fig 1*C*). Additional studies revealed that the epidermal ODC activity slowly decreased following discontinuation of 4OHT treatment. Two-thirds of the epidermal ODC activity was decreased by 2 wk after 4OHT discontinuation, but the basal level of ODC activity remained elevated compared with the basal level of activity before 4OHT treatment (data not shown). Comparative analysis of the ODC activity in the skin of the three lines of ODCER transgenic mice and the previously described K6/ODC transgenic mouse, in which the ODC transgene is directed to the skin by a K6 promoter, shows that cutaneous ODC activity is typically induced to higher levels in ODCER2 transgenic mice treated for 21 d with 4OHT compared with that seen in constitutively expressed ODC in the K6/ODC transgenic mouse (Fig 1*B*).

Involucrin is expressed in the suprabasal layers of the epidermis and the inner root sheath of hair follicles. We confirmed the suprabasal localization of the involucrin-regulated ODC protein and the ER ligand-binding domain of the encoded ODCER fusion protein by immunohistochemistry (Fig 3). As previously seen (Gilmour *et al*, 1986), basal levels of endogenous ODC protein are virtually non-detectable by immunohistochemical staining in wild-type mouse epidermis (Fig 3*A*). ODC staining, however, was detectable in the suprabasal cells of the interfollicular epidermis and in the inner root sheath of ODCER2 hair follicles both with and without treatment with 4OHT (Fig 3*B*, *C*). Furthermore, staining for ER protein was detected in the same suprabasal epidermal cells of ODCER2 mice with and without 4OHT but was not detected in normal littermates treated with 4OHT (Fig 3*D*–*F*). This staining pattern was similar to that found in other transgenic mouse models encoding the c-Myc-ER^{TAM} protein in which the c-Myc-ER^{TAM} protein is expressed constitutively in the absence of the 4OHT ligand but sequestered in an inactive state with the heat-shock-protein 90 (HSP90) (Pelengaris *et al*, 1999, 2002). Treatment with 4OHT leads to HSP90 dissociation and activation of the ER-chimeric protein.

Analyses of lysates prepared from separated epidermal and dermal tissue revealed that the induction of ODC activity following 14 d of topical 4OHT treatments was specific in the epidermis and not in the underlying dermis (Fig 4*A*).

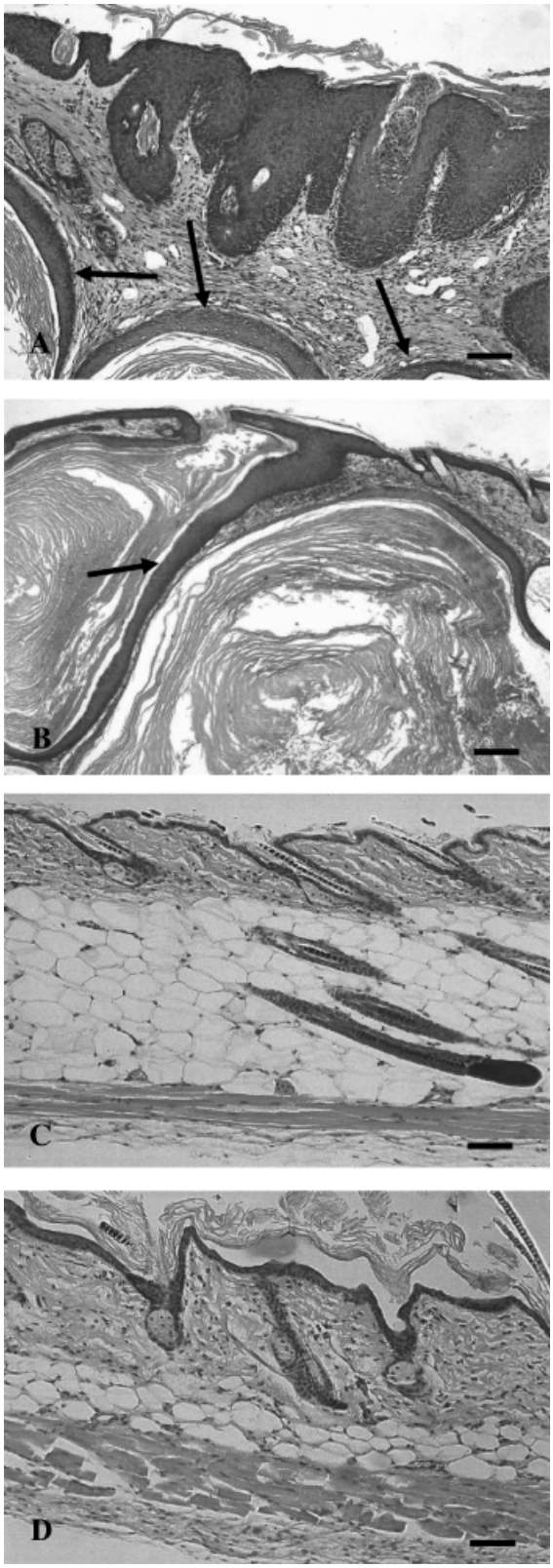

Figure 2
High basal levels of suprabasal ornithine decarboxylase (ODC) activity lead to hair loss and dermal cyst formation in ODCER1 and ODCER3 transgenic mice. Comparison of skin from (*A*, *B*) 11-mo-old ODCER3 transgenic mice never treated with 4-hydroxytamoxifen (4OHT); (*C*) a 15-mo-old ODCER2 transgenic mouse never treated with 4OHT; and (*D*) a 3-mo-old ODCER2 transgenic mouse topically treated with 4OHT for 21 d. Note the huge dermal cysts (*arrows*) and focal areas of epidermal hyperplasia in the ODCER3 transgenic skin. Hematoxylin & eosin-stained sections. *Scale bars*: (*A*) 50 μm, (*B*) 100 μm, (*C*) 50 μm, (*D*) 40 μm.

The 4OHT-stimulated increase in ODC enzyme activity was accompanied by increased levels of putrescine in the 4OHT-treated epidermis compared with vehicle control-treated ODCER2 epidermis, but not in the dermis (Fig 4*B*). There were no significant changes in spermidine or spermine levels following 2 wk of 4OHT treatment in either the epidermis or dermis of ODCER2 transgenic mice (data not shown). These results demonstrate the suprabasal activation of the ODCER protein and subsequent elevated ODC enzyme activity and putrescine levels in the adult epidermis of the ODCER2 transgenic mouse line following topical 4OHT treatment.

***De novo* induction of suprabasal epidermal ODC stimulates proliferation** To determine the effect of induction of ODC enzyme activity on epidermal proliferation, *in vivo* bromodeoxyuridine (BrdU) labeling was performed in adult ODCER2 transgenic mice following 7 and 21 d 4OHT treatment. Although 1 wk of topical application of 4OHT resulted in no significant increase in BrdU-stained nuclei (data not shown), continued 4OHT treatment for 21 d resulted in a significantly increased labeling index (Fig 5), with a 4-fold increase compared with non-treated transgenic skin or 4OHT-treated normal littermate skin. Labeled nuclei were detected only in the basal layer of interfollicular epidermal cells and not in the suprabasal compartment. Despite the increased proliferation index, there were no obvious gross changes in the skin. Histological examination of ODCER2 transgenic skin following 21 d of 4OHT treatment revealed an epidermis only one to two additional cell layers thick with infrequent small areas of mild epidermal hyperplasia.

Since there was no marked epidermal hyperplasia following transient activation of ODC, we analyzed the ODCER2 transgenic skin for epidermal differentiation markers following daily topical treatment with 4OHT for 3 wk. Although immunoblot analysis of skin lysates revealed increased expression of the proliferation marker, proliferating cell nuclear antigen (PCNA), in 4OHT-treated transgenic skin compared with non-treated transgenic skin and normal littermate skin, the expression of markers for commitment to differentiation, including K1, K10, and involucrin, was increased in 4OHT-treated transgenic skin as well (Fig 6). TUNEL staining revealed no increased apoptosis in the skin of ODCER2 transgenic mice following treatment with 4OHT (data not shown). These data suggest that transient activation of ODC activity stimulates proliferation only in keratinocytes that are not committed to differentiate, i.e. cells in the basal compartment of the epidermis. This ODC proliferative stimulus, however, results in a mild expansion of the proliferative compartment that is compensated by a concomitant expansion of the differentiated compartment in 4OHT-treated transgenic skin. Thus, expression of differentiation markers is increased as well.

To further determine the effect of ODC overexpression on differentiation markers in keratinocytes, primary keratinocytes were isolated from ODCER2 transgenic mouse skin and normal littermate skin. In this cultured system, keratinocytes proliferate when maintained in low-calcium medium (0.05 mM calcium), but terminally differentiate and cease to proliferate after calcium is raised to 0.14 mM in the medium. Expression of K1 and K10 marks an early event that occurs

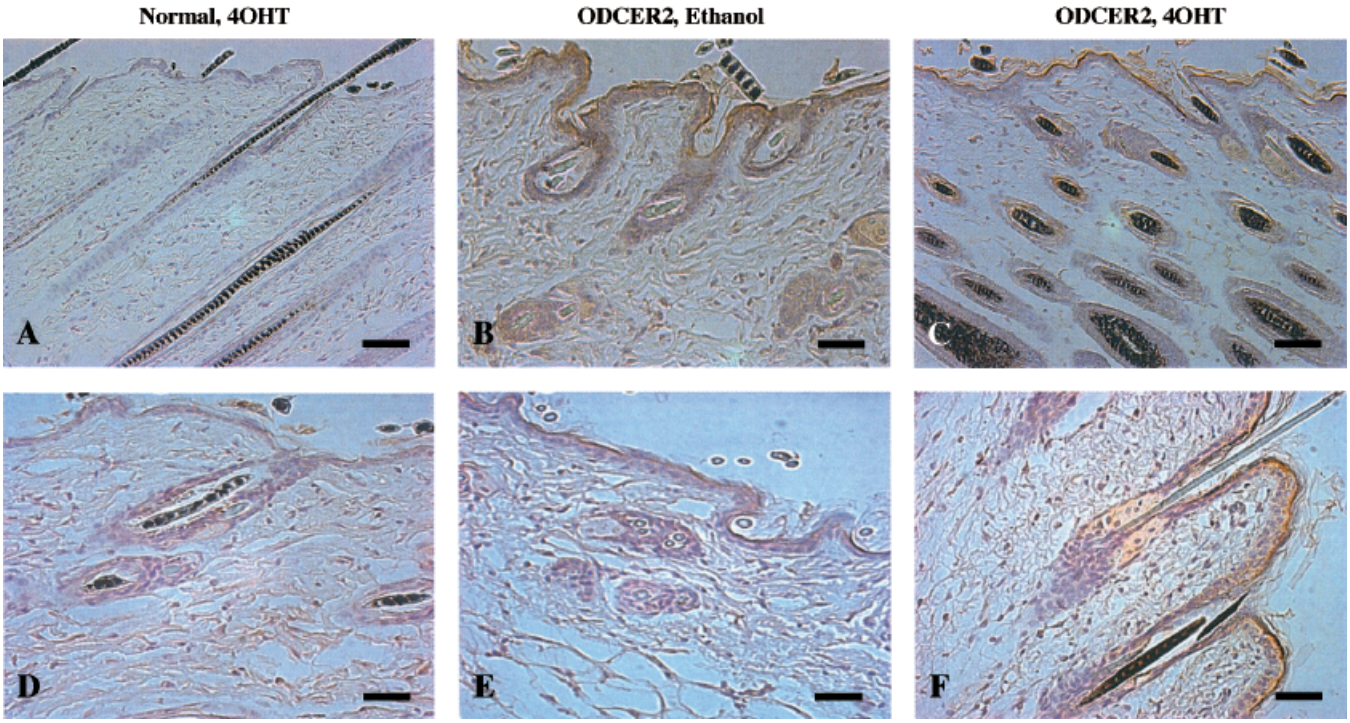

Figure 3
Suprabasal induction of ornithine decarboxylase (ODC) and estrogen receptor (ER) protein in ODCER2 transgenic mice. Skin sections from normal wild-type littermates (*A*, *D*) and ODCER2 transgenic mice topically treated with the vehicle control ethanol (*B*, *E*) or 4-hydroxytamoxifen (4OHT) (*C*, *F*) for 21 d were immunohistochemically stained using an anti-ODC antibody (*A–C*) or an anti-ER antibody (*D–F*). Sections were counterstained with hematoxylin. *Scale bars*: (*A–D*) 50 μm; (*E*, *F*) 40 μm.

as keratinocytes commit to differentiate terminally, followed by expression of loricrin (Yuspa *et al*, 1989). ODC activity was induced in ODCER2 transgenic keratinocytes following treatment with 1.0 μM 4OHT. In addition, ODC activity was higher in the ODCER transgenic keratinocytes following the switch to 0.14 mM calcium compared with that at low calcium because of calcium activation of the involucrin promoter of the ODCER transgene. Thus, the highest ODC enzymatic activity was present in the ODCER transgenic keratinocytes cultured in 0.14 mM calcium and treated with 4OHT. As expected, differentiation markers such as K1 were induced 24 h following the calcium switch in wild-type keratinocytes (Fig 7*A*). Induction of ODC in ODCER2 transgenic keratinocytes partially blocked the calcium induction of K1 and K10 (Fig 7*A*). At 48 h following the calcium switch, later differentiation markers involucrin and loricrin were induced in both 4OHT-treated and non-treated normal and transgenic keratinocytes (Fig 7*A*). Elevated levels of ODC and polyamines, however, did not rescue the keratinocytes from a calcium-induced block in DNA synthesis (Fig 7*B*). These results indicate that elevated levels of ODC activity do not inhibit the commitment of keratinocytes to differentiate terminally, but the differentiation program is moderately altered as evidenced by a partial block in the induction of early differentiation markers in response to a calcium switch.

Suprabasal ODC expression in skin triggers angiogenesis We examined the effects of transient suprabasal epidermal ODC expression on the blood vessel density in the underlying dermis. Mice received an injection with fluorescein isothiocyanate (FITC)-lectin to label vessels with active blood flow. Because of binding of the lectin to endothelial cells, the fluorescent staining clearly outlined functional vessels with an open lumen (Fig 8*A*). Quantitation of FITC-lectin-labeled endothelial cells revealed marked increases in blood vessel density in 4OHT-treated ODCER2 transgenic skin compared with non-treated transgenic skin or wild-type littermate skin (Fig 8*B*). Increased vascularization of transgenic skin was observed as early as 2 wk of daily 4OHT treatments. In addition, sections of skin from FITC-lectin-injected mice were immunofluorescent stained for α-smooth muscle actin (α-SMA)-positive pericytes. Pericytes were found to be associated in a uniform layer around many of the blood vessels as indicated by red fluorescence for pericytes alone or yellow double fluorescence for vessels where green FITC-lectin-stained endothelial cells overlapped with red-stained pericytes (Fig 8*A*). Thus, 4OHT induction of ODC activity in ODCER2 transgenics increased the vascularization of the skin, and many of these newly formed microvessels were able to recruit pericytes.

Elevated epidermal ODC activates keratinocytes ODC and polyamines are elevated in skin undergoing tissue remodeling in such processes as wound healing and tumor formation. These conditions are characterized by increased production of cytokines such as interleukin (IL)-1 (Maas-Szabowski *et al*, 2000; Murphy *et al*, 2000; Freedberg *et al*, 2001) and altered expression of intermediate filaments such as K6 and K16 (Freedberg *et al*, 2001; Komine *et al*, 2001). Since tissue remodeling in skin includes increased proliferation and angiogenesis and altered gene expression, we

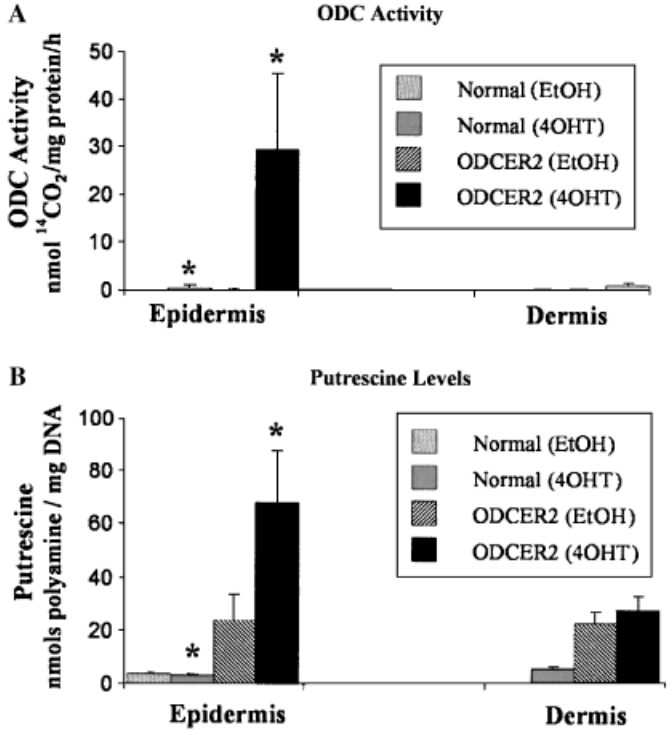

Figure 4
Induction of ornithine decarboxylase (ODC) activity and putrescine in the epidermis but not in the dermis of 4-hydroxytamoxifen (4OHT)-treated ODCER2 transgenic mice. ODCER2 transgenic mice and their normal wild-type littermates were topically treated with 4OHT (daily administration of 1.0 mg 4OHT per 0.1 mL ethanol) or the ethanol vehicle control for 14 d. Lysates of epidermal and dermal tissue from ODCER2 transgenic mouse skin and non-transgenic littermate (normal) mouse skin were prepared 24 h after the last treatment and assayed for *A*) ODC enzyme activity and *B*) putrescine levels. ODC enzyme activity was expressed as nmol $^{14}CO_2$ per h per mg protein and putrescine levels were expressed as nmol per mg DNA. Values are means $\pm$ SD, and are representative of three separate experiments with at least four mice per treatment group. $^{*}p < 0.02$.

examined whether ODC overexpression in the skin of ODCER2 transgenic mice leads to altered expression of genes in a manner similar to that seen in wound healing. Therefore, we screened ODC overexpressing epidermal cells in ODCER2 transgenic mice for expression of angiogenic factors that could affect the endothelial cells directly. Use of Panomics cytokine and angiogenesis antibody arrays and immunoblot analyses revealed increased expression of IL-1β with higher ODC activity in the skin (Fig 9*A*). ELISA assays confirmed that IL-1β levels were increased in the skin of 4OHT-treated ODCER2 transgenic mice (Fig 9*B*).

Another marker of hyperproliferative, activated epidermis is the induced expression of K6 and extracellular matrix protein such as tenascin-C (Natali *et al*, 1991; Latijnhouwers *et al*, 1997). RT-PCR analysis confirmed that transient induction of ODC in ODCER epidermis leads to increased levels of K6β but not K6α mRNA expression (Fig 10*A*). K6 expression was also increased on the protein level both in epidermal and dermal extracts of skin from ODCER2 transgenic mice treated for 7 d with 4OHT (Fig 10*B*). Immunohistochemical staining revealed increased K6 protein in the intrafollicular epidermis of 4OHT-treated ODCER2 skin (Fig 11*A*, *B*). In addition, focal areas of dermal tenascin-

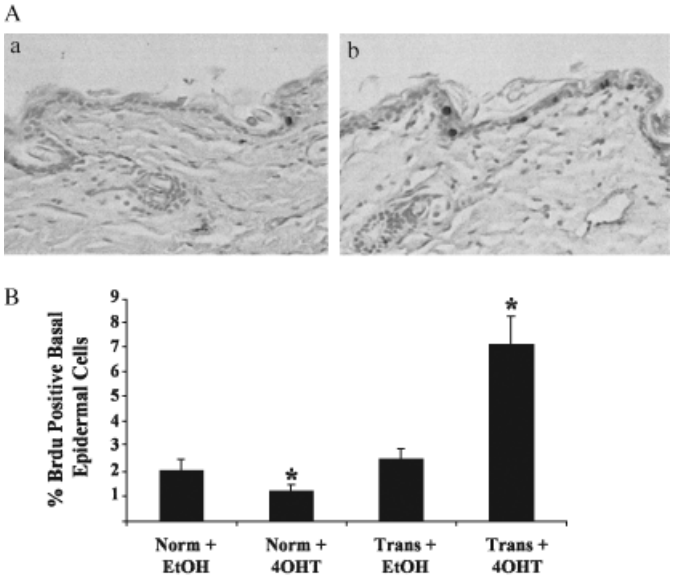

Figure 5
Transient suprabasal induction of ornithine decarboxylase (ODC) increases the proliferation of basal cells in the skin of ODCER2 transgenic mice. ODCER2 transgenic mice and their normal wild-type littermates were topically treated with the ethanol vehicle control or with 4-hydroxytamoxifen (4OHT) for 21 d ($n = 3$ or more animals for each treatment group). Mice received injections of bromodeoxyuridine (BrdU) at a dose of 100 μg per g body weight 2 h before sacrifice. Paraffin-embedded sections were immunostained using an anti-BrdU antibody. (*A*) Immunostained skin sections from ODCER2 transgenic mice that were topically treated 21 d with (*A*) ethanol or (*B*) 4OHT. Note that BrdU-stained nuclei were only detected in the basal cell layer of the epidermis. *Scale bars*: 50 μm. (*B*) BrdU-positive cells per 1000 cells in the basal layer were counted in three to five sections for each treatment group. The percentage of BrdU-positive cells expressed as the mean + SD. $^{*}p < 0.01$.

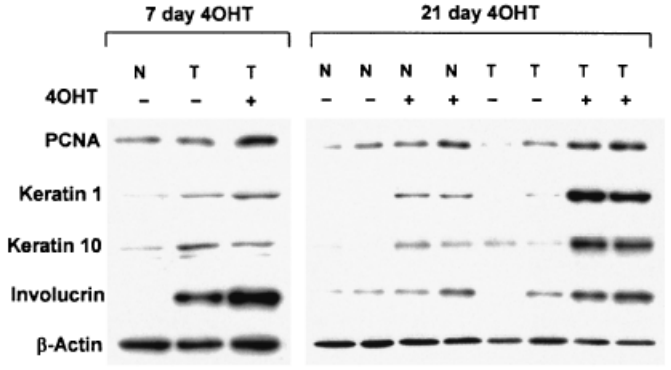

Figure 6
Ornithine decarboxylase (ODC) induction stimulates both proliferation and differentiation in ODCER2 transgenic mouse skin. RIPA lysates of total skin tissue from ODCER2 mice (*T*) and their normal wild-type littermates (N) were prepared after daily topical treatment with 1.0 mg 4-hydroxytamoxifen (4OHT) per 100 μL ethanol or the ethanol vehicle control for 7 or 21 d. Equal amounts of protein were loaded and resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The proliferation marker PCNA and the differentiation markers keratin 1, keratin 10, and involucrin were detected by immunoblotting. Banding pattern is representative of immunoblots with at least three mice per treatment group. The blot was reprobed for β-actin to monitor for equal protein loading.

C staining proximal to the epidermal basement membrane were observed under sections of epidermis that exhibited mild hyperplasia in ODCER2 transgenic skin following 4OHT

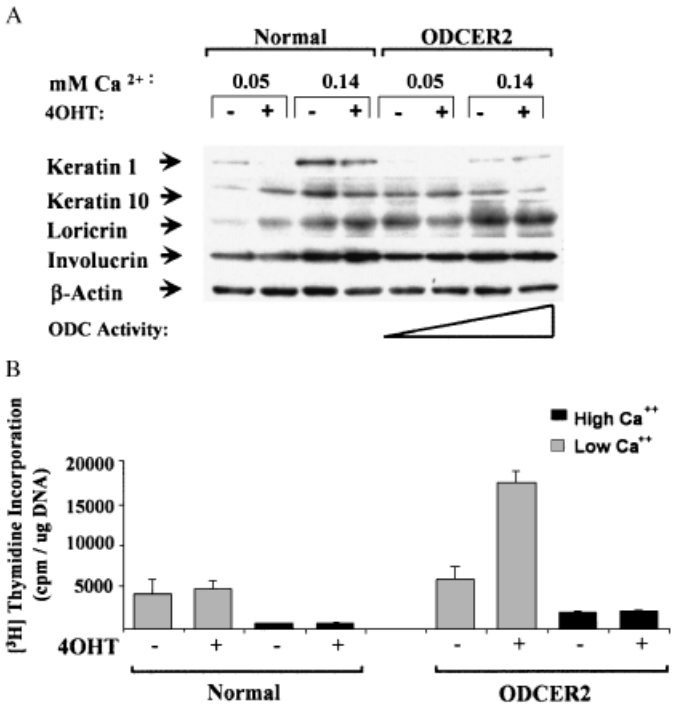

Figure 7
Elevated ornithine decarboxylase (ODC) activity alters calcium-induced differentiation in primary keratinocytes from ODCER2 transgenic mouse skin. Primary keratinocytes were isolated from the skin of ODCER2 mice and their normal littermates and treated with 1.0 μM 4-hydroxytamoxifen (4OHT) or the ethanol vehicle control for 48 h. At the same time, the calcium concentration was raised to 0.14 mM in the medium of half the keratinocytes to trigger differentiation. (*A*) RIPA lysates were prepared 48 h after the calcium switch and equal amounts of protein were resolved by SDS-PAGE. Keratin 1, keratin 10, loricrin, involucrin, and β-actin proteins were detected by immunoblotting. (*B*) Cell proliferation was assessed by the amount of [^{3}H]thymidine incorporation (c.p.m.) normalized to micrograms DNA. Values are means ± SD of at least three samples, and are representative of three separate experiments.

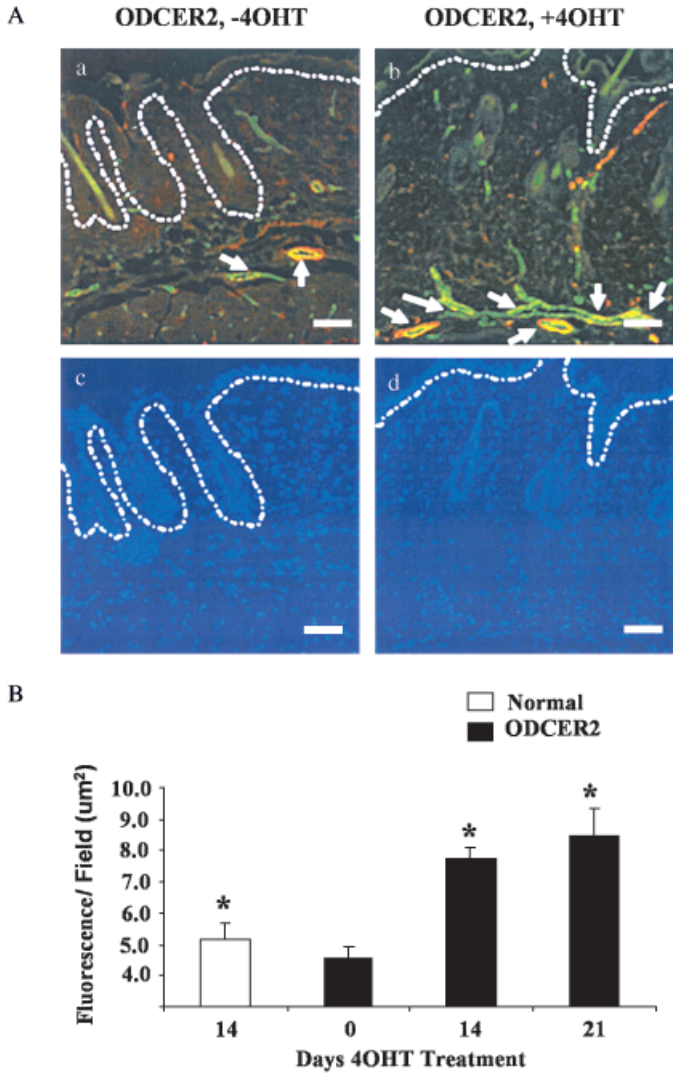

Figure 8
Induction of ornithine decarboxylase (ODC) activity stimulates vascularization in the skin of ODCER2 mice. ODCER2 transgenic mice (*closed bars*) and their normal wild-type littermates (*open bar*) were treated with 4-hydroxytamoxifen (4OHT) or the ethanol vehicle control for 14 or 21 d. Mice were injected with 0.1 mL fluorescein isothiocyanate (FITC)-lectin (1 mg per mL) via the tail vein 5 min before sacrifice to visualize endothelial cells of functional blood vessels. (*A*) Skin tissue was fixed in 4% *p*-formaldehyde, paraffin-embedded, and sections were immunohistochemically stained for α-smooth muscle actin (α-SMA) using a Cy3-labeled secondary antibody, covered with DAPI mounting medium, and examined by fluorescence microscopy. Both FITC-lectin-stained blood vessels (*green*) and α-SMA-positive pericytes (*red*) are shown in overlaid fluorescent images (*a*, *b*) of skin from ethanol-treated ODCER2 mice (*a*, *c*) and of skin from 4OHT-treated ODCER2 mice (*b*, *d*). DAPI (*blue*) images are shown for the same view to identify all nuclei (*c*, *d*). The basement membrane is marked with the white dashed line. Arrows point to overlap (*yellow*) of pericytes and endothelial cells of blood vessels. *Scale bars*: 40 μm. (*B*) The amount of endothelial cells (FITC) was quantified by calculating the fluorescein intensity/field of view using a × 10 objective. The data represent the mean fluorescent area of five fields examined for each tissue section + SD, with three to five separate skin samples from different mice analyzed/each treatment group. *$p < 0.02$.

treatment (Fig 11*C*, *D*). Since IL-1β, K6, and tenascin-C expression are induced in hyperproliferative skin following wounding (Singer and Clark, 1999), ODC induction of these genes implies that transient induction of ODC in quiescent adult epidermis is sufficient to "activate" keratinocytes.

Spontaneous tumor formation Unlike the ODCER2 transgenic line, the ODCER1 and ODCER3 transgenic lines exhibit not only much higher ODC enzyme activity in the non-induced skin leading to hair loss but also develop focal areas of epidermal hyperplasia by 6 mo of age (Fig 2*A*, *B*). Moreover, some epithelial cells lining dermal cysts are moderately hyperplastic in the skin of ODCER3 mice following hair loss. In contrast, older, non-treated ODCER2 mice or ODCER2 mice treated with 4OHT for 3 wk demonstrate no hair loss and only mild epidermal hyperplasia with short-term treatment with 4OHT (Fig 2*C*, *D*). By 10 mo of age, ODCER1, ODCER2, and ODCER3 transgenic mice develop cyst-like growths on the tail skin without 4OHT induction (Fig 12*A*). In addition, occasionally larger keratin-filled skin tumors appear on other parts of the body of ODCER1 and ODCER3 transgenic mice but not the haired ODCER2 mice. These tumors are benign, highly differentiated cyst-like growths that sometimes regress (Fig 12*B*, *C*).

To examine whether suprabasal expression of ODC is sufficient to promote the development of spontaneous skin tumors in the presence of an activated *ras*, ODCER3 transgenic mice were bred with Tg.AC transgenic mice possessing the v-Ha-*ras* transgene (Leder *et al*, 1990). Double

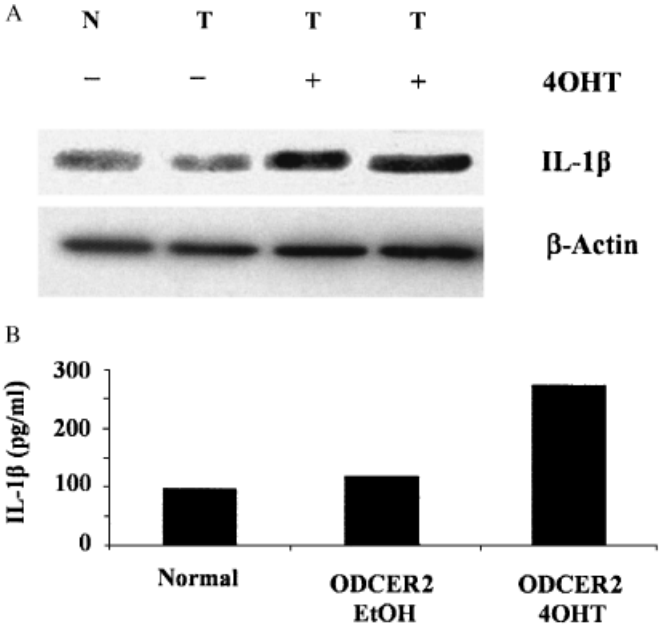

Figure 9
Induction of ornithine decarboxylase (ODC) activity stimulates interleukin (IL)-1β expression in ODCER2 transgenic skin. ODCER2 transgenic mice and their normal wild-type littermates were treated topically with 4-hydroxytamoxifen (4OHT) or the ethanol vehicle control for 21 d. (*A*) RIPA lysates were subjected to western blotting using antibodies against IL-1β and β-actin. (*B*) IL-1β protein levels in skin lysates from ODCER2 transgenic mice (*T*) and their normal littermates (*N*) were also quantitated by ELISA assay.

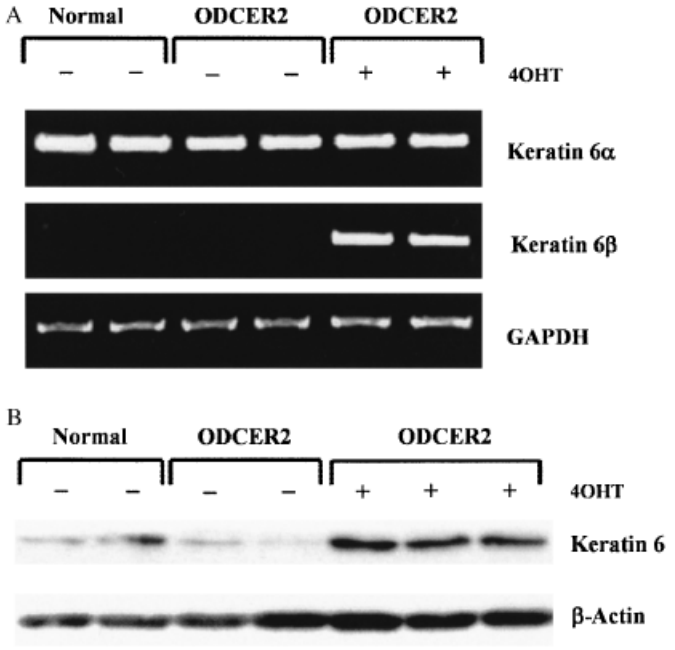

Figure 10
Upregulation of keratin 6 in the skin of 4-hydroxytamoxifen (4OHT)-treated ODCER2 transgenic mice. (*A*) Expression of keratin K6α and K6β mRNA was examined by reverse transcription-PCR using RNA extracted from the skin (including both the epidermis and dermis) of ODCER2 transgenic mice and their normal littermates topically treated for 7 d with 4OHT or the ethanol vehicle control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was included as an internal control. (*B*) ODCER2 transgenic mice and normal wild-type littermates were treated topically with 4OHT for 7 d, and keratin 6 protein expression was determined in epidermal and dermal RIPA lysates by immunoblot analysis. The blot was reprobed for β-actin to monitor for equal protein loading.

transgenic mice developed spontaneous malignant squamous cell carcinomas between 7 and 12 wk of age without prior 4OHT treatment (Fig 12*D*). In contrast, ODCER2 transgenic mice bred with Tg.AC transgenic mice did not form skin tumors unless treated with 4OHT. These tumors appeared to develop from hair follicles (Fig 12*E*) and to form keratoacanthomas (Fig 12*F*). Thus, suprabasal epidermal ODC overexpression leads to a mild epidermal hyperplasia that can, with time, result in the spontaneous formation of benign tumors but can also rapidly lead to malignant carcinomas when combined with a mutated *ras* oncogene.

Discussion

This study demonstrates that *de novo* induction of ODC activity in adult mouse skin stimulates both epidermal proliferation and angiogenesis. Healthy keratinocytes normally proliferate slowly in the basal layer and differentiate as they move up through the spinous, granular, and cornified layers in the epidermis (Eckert *et al*, 1997; Mischke, 1998). In response to wounding or many pathologic conditions, however, keratinocytes are activated to become migratory, hyperproliferative cells producing growth factors and cytokines that, in turn, activate underlying dermal fibroblasts and endothelial cells (Romero *et al*, 1997; Maas-Szabowski *et al*, 2000; Freedberg *et al*, 2001). Keratinocyte activation appears to be initiated by IL-1, and these activated cells are characterized by upregulation of K6 and K16 (Kupper, 1990; Tomic-Canic *et al*, 1998; Freedberg *et al*, 2001; Komine *et al*, 2001). Our results demonstrating increased proliferation, neovascularization, and elevated expression of proteins such as IL-1β, K6, and tenascin-C in 4OHT-treated ODCER2 transgenic mouse skin indicate that elevated polyamine levels activate keratinocytes and stimulate angiogenesis in the dermal layer in a manner similar to skin responding to a wound.

ODCER2 transgenic mice fortuitously demonstrated a low basal level of ODC activity similar to wild-type mice. As a result, ODCER2 mice displayed a normal skin phenotype with no hair loss or formation of dermal cysts as seen in the ODCER1 and ODCER3 transgenic lines. The basal ODC enzyme activity did not correspond to the expression of ODCER protein in the three transgenic lines. It is likely that the low ODC basal activity observed in the skin of ODCER2 transgenic mice is associated with a different integration site of the ODCER transgene in the mouse genome that influences other interacting proteins that modulate ODC activity. The ODCER2 transgenic mouse is novel in that it is the first mouse model in which the effects of *de novo* induction of polyamine biosynthesis can be studied in normal adult skin. Indeed, ODC enzyme activity is specifically induced in ODCER2 mouse epidermis following topical treatment with 4OHT.

Although the proliferative index was increased, elevated levels of epidermal ODC activity over 3 wk time did not lead to significant epidermal hyperplasia as is seen in wound healing. Indeed, differentiation markers as well as proliferation markers were upregulated in 4OHT-treated ODCER2

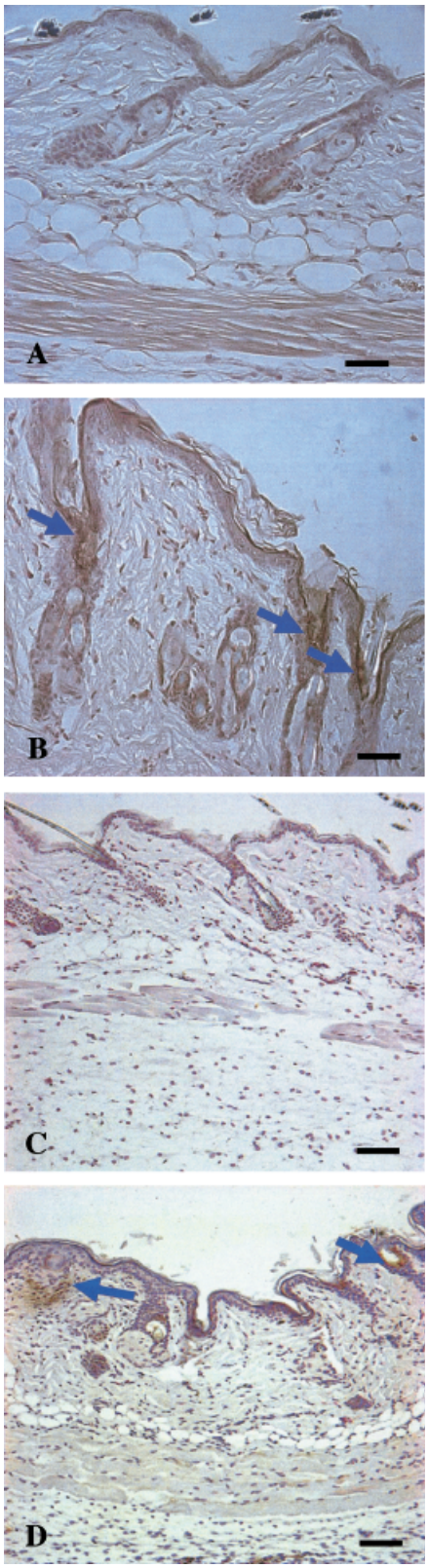

Figure 11
Elevated levels of epidermal ornithine decarboxylase (ODC) activity induce focal areas of keratin 6- and tenascin-C-stained cells in transgenic skin. ODCER2 transgenic mice were topically treated for 21 d with 4-hydroxytamoxifen (4OHT) (*B*, *D*) or the ethanol vehicle control (*A*, *C*). Paraffin skin sections were immunostained using a polyclonal antibody that recognizes both the α and β isoforms of keratin 6 (*A*, *B*) or using a polyclonal antibody against tenascin-C (*C*, *D*). Arrows point to stained cells. *Scale bars*: 50 μm.

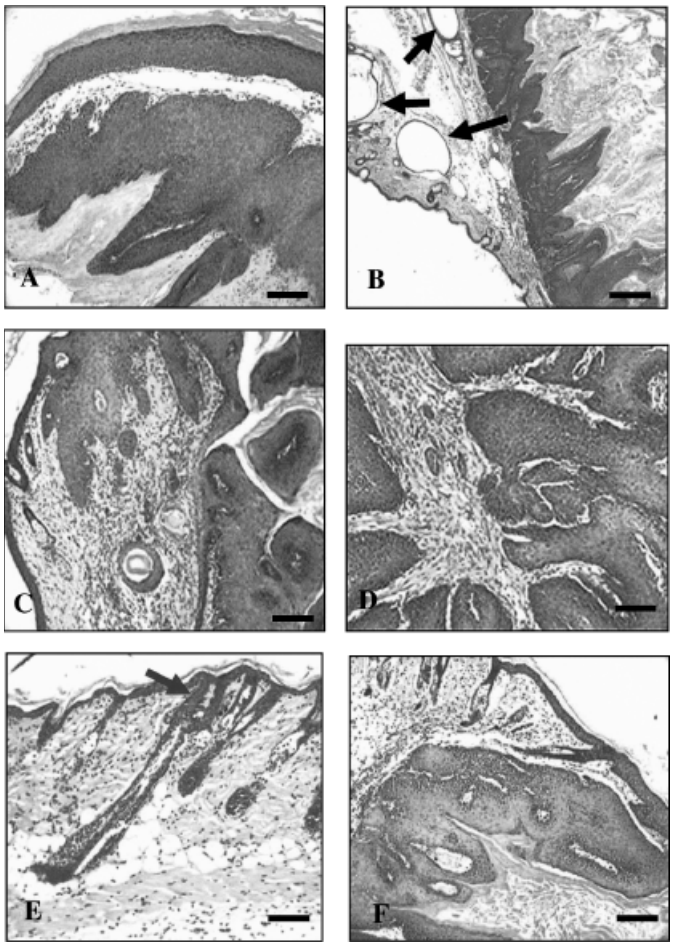

Figure 12
Tumor formation in ODCER transgenic mice. ODCER3 transgenic mice (*A–C*) develop spontaneous, benign skin tumors by 6–10 mo of age. Black arrows point to follicular cysts that develop in the dermis of ODCER3 mice following hair loss. Double transgenic mice, generated by breeding ODCER3 transgenic mice with Tg.AC v-Ha-*ras* transgenic mice, develop spontaneous malignant carcinomas between 7 and 12 wk of age (*D*). In contrast, skin tumors develop in ODCER2 transgenic mice crossed with Tg.AC v-Ha-*ras* transgenic mice (*E*, *F*) following 5 wk of topical 4-hydroxytamoxifen treatment with the skin tumors arising from hyperplasia of perifollicular cells (*blue arrow*) to form keratoacanthomas (*F*). hematoxylin & eosin-stained sections. *Scale bars*: (*A*, *C–F*) 50 μm, (*B*) 150 μm.

transgenic skin. Furthermore, induction of ODC activity did not rescue primary ODCER2 keratinocytes from a calcium-triggered DNA synthesis block, as measured by [^{3}H]thymidine incorporation. *De novo* induction of suprabasal ODC activity did not block the commitment of this growing epidermal compartment to differentiate, and BrdU-labeled nuclei appeared in only the basal layer of the epidermis yielding no dramatic epidermal hyperplasia. As has previously been reported in wound-healing studies in wild-type mice (Mansbridge and Knapp 1987; Paladini *et al*, 1996), the induction of K6 expression in the interfollicular epidermis correlated with less K1 and K10 induction in 4OHT-treated ODCER2 keratinocytes. A major difference between Myc or Ras-inducible transgenic mouse models (Pelengaris *et al*, 1999; Tarutani *et al*, 2003) and the ODCER2 transgenic model is that induction of ODC does not appear to inhibit differentiation of post-mitotic keratinocytes. In contrast, transient induction of c-Myc in the suprabasal layers of the

epidermis blocks differentiation and also triggers proliferation of post-mitotic epidermal cells that have already begun their differentiation program (Pelengaris *et al*, 1999). Unlike ODCER2 transgenic mice where ODC overexpression stimulates both proliferation and differentiation, Ras and Raf activation suppress *in vivo* epidermal differentiation (Tarutani *et al*, 2003).

Our results show that elevated levels of polyamines activate keratinocytes, but do not transform keratinocytes over a short time as has been reported for other transgenic mouse models in which c-Myc, Ras, or Raf were induced in the epidermis (Pelengaris *et al*, 1999; Tarutani *et al*, 2003). Sustained c-Myc stimulation of epidermal proliferation resulted in benign skin tumors and marked angiogenesis (Pelengaris *et al*, 1999). Similarly, the transient activation of Ras or its downstream effector Raf in mouse epidermis promoted the hyperproliferative phenotype characteristic of benign papillomas (Tarutani *et al*, 2003). Although 21 d of 4OHT induction did not lead to skin tumors in ODCER2 mice, epidermal hyperplasia and spontaneous benign skin tumors developed in ODCER1 and ODCER3 mice that possess higher basal epidermal ODC activity compared with ODCER2 mice. Thus, elevated levels of epidermal ODC activity over a longer time lead to the development of benign skin tumors. Interestingly, administration of the ODC inhibitor DFMO to Inv/MycER transgenic mice partially inhibited the formation of tumors (S. Pelengaris, personal communication). ODC is known to be transactivated by c-myc (Bello-Fernandez *et al*, 1993; Ben-Yosef *et al*, 1998), and topical 4OHT treatment reportedly upregulated ODC mRNA in the skin of Inv/MycER transgenic mice (Pelengaris *et al*, 1999). Although Myc transactivates a number of genes in addition to ODC (Ben-Yosef *et al*, 1998), these observations suggest that upregulation of ODC and polyamines plays an essential role in Myc-induced skin tumorigenesis. In view of our data demonstrating the rapid development of spontaneous carcinomas in ODC/Ras double transgenic mice and the chemopreventive activity of DFMO in tumor formation in Inv/MycER transgenic mice, elevated polyamine levels appear to cooperate with altered expression of a signaling pathway that is perhaps in common to and triggered by both Myc and Ras activation to promote skin tumorigenesis. Moreover, ODC-induced activation of epidermal cells not only promotes early stages of skin tumorigenesis but also later stages requiring invasion to surrounding tissues since squamous cell carcinomas spontaneously develop in double transgenic mice expressing both an ODC and a v-Ha-*ras* transgene.

Previous studies using the K6/ODC transgenic mouse, in which a K6 promoter drives the expression of ODC in the outer root sheath cells of hair follicles, show that continuous increased expression of epidermal ODC leads to increased vascularization of the underlying dermis (Lan *et al*, 2000). K6/ODC mouse skin is, however, a gross distortion of normal skin architecture because of disruption of the hair cycle resulting in the development of dermal cysts as the hair follicles degenerate. Although the K6/ODC transgenic mouse is a useful model, the altered skin morphology may complicate the interpretation of biological effects resulting from high polyamine levels. Since the epidermal cells that line the follicular cysts in K6/ODC transgenic mice express very high levels of ODC, we questioned whether the increased vascularization of the K6/ODC dermis is influenced by the close proximity of ODC overexpressing cells and endothelial cells found in the dermis. One of the unique strengths of the ODCER2 transgenic mouse model is that ODC activity is induced *de novo* in adult skin, thus maintaining a normal skin phenotype without the hair loss or the resulting development of follicular cysts that is characteristic of other transgenic mice with altered polyamine metabolism. Although 4OHT treatment increased putrescine levels in only the epidermis and not in the underlying dermis, it is possible that the increased vascularization of ODCER2 transgenic skin may, in part, result from polyamines diffusing to the dermal compartment and directly activating endothelial cells.

Most likely the increased vascularization in the dermis results from angiogenic factors that are produced and secreted by keratinocytes as a consequence of increased ODC activity. As part of a normal response to damage or wounding, proliferating keratinocytes secrete angiogenic factors (Martin, 1997). As is found in wound healing when keratinocytes first become activated by the release of IL-1 (Freedberg *et al*, 2001), this study reveals that induction of epidermal ODC increases the levels of IL-1. IL-1β has been shown to be a proangiogenic factor in solid tumors (Voronov *et al*, 2003). Moreover, IL-1β can upregulate VEGF and downregulate the anti-angiogenic factor angiopoietin-1, thus promoting neovascularization of a tissue (Fan *et al*, 2004). Cytokines released by activated keratinocytes can recruit inflammatory cells including macrophages and mast cells, which have been shown to express MMP-2 and MMP-9 coinciding with the angiogenic switch in pre-malignant skin lesions (Coussens *et al*, 2000). Although focal areas of 4OHT-treated ODCER2 skin showed increased numbers of mast cells in the underlying dermis compared with non-treated ODCER2 skin (data not shown), we have no evidence that increased inflammatory cells are responsible for the increased angiogenesis induced by elevated epidermal ODC activity. Since angiogenesis is determined by the balance of both pro-angiogenic and anti-angiogenic factors, work is underway to determine polyamine-modulated factors that are responsible for the increased vascularization in ODCER2 skin.

Another consequence of ODC induction in adult skin is the early induction of K6, which is associated with hyperproliferative conditions. It has been suggested that changes in the composition of the cytoskeleton may enhance keratinocyte motility and/or hyperproliferation (Wawersik and Coulombe, 2000; Komine *et al*, 2001). On the RNA level, the K6β isoform is selectively induced when polyamine levels are increased. It is interesting that previous studies have shown that whereas the K6α isoform mRNA predominates in the intact epithelia, the K6β mRNA is induced to a significantly greater extent following injury or treatment of skin with phorbol esters (Takahashi *et al*, 1998; Rothnagel *et al*, 1999). IL-1 has been reported to induce the transcription of K6 as part of the activation of keratinocytes in pathologic conditions such as wound healing, tumors, psoriasis, and other inflammatory disorders (Komine *et al*, 2001).

Tenascin-C, a large complex protein of the extracellular matrix, is expressed transiently at high levels during devel-

opment and then strongly upregulated during tissue remodeling processes such as tumorigenesis and wound healing (Chiquet-Ehrismann *et al*, 1986; Sakakura and Kusano, 1991; Hanamura *et al*, 1997; Jones and Jones, 2000). That increased polyamine biosynthesis is responsible for the localized detection of tenascin-C in 4OHT-treated ODCER2 transgenic skin is corroborated by our observation that elevated levels of polyamines stimulate tenascin-C expression in cultured ODC-overexpressing primary keratinocytes (data not shown). Dermal tenascin-C expression has been shown to be strongly induced in dermal tissue adjacent to hyperproliferative epidermis during wound healing (Latijnhouwers *et al*, 1996). In addition, tenascin-C has been implicated in angiogenesis, the regulation of VEGF (Tanaka *et al*, 2004), and in modulating cell adhesion and cell motility (Swindle *et al*, 2001; Midwood *et al*, 2004; Mukaratirwa *et al*, 2004). Thus, as a consequence of increased polyamine biosynthesis, keratinocytes become more proliferative and release cytokines and extracellular protein such as tenascin-C that, in turn, could activate stromal cells to stimulate angiogenesis.

This study reveals that the expression of many characteristic markers of wound healing in skin, including IL-1, K6, and tenascin-C, is induced in non-wounded skin with high levels of epidermal polyamines. Polyamine activation of keratinocytes and underlying stromal cells is an early event in the tumor process that would create a more permissive microenvironment for tumor development. The ODCER2 transgenic mouse model is valuable in that it can be used to further investigate how these early changes in polyamine levels in a normal skin tissue can affect the expansion of dormant, genetically altered epidermal cells to a malignant phenotype.

Materials and Methods

Construction of involucrin-ODCER transgene The hormone-binding domain of the modified murine ER was fused in frame at the 3′ end of a cDNA encoding amino acids 1–426 of a truncated murine ODC protein (Littlewood *et al*, 1995), previously shown to undergo less rapid intracellular protein degradation (Ghoda *et al*, 1989), in the plasmid pcDNA3.1 (Clontech, Palo Alto, California). The involucrin promoter construct pH3700-pL2 (Carroll *et al*, 1993) consists of 2.5 kb of the human involucrin upstream region, the involucrin intron, an SV40 intron, and an SV40 polyadenylation sequence. The ODCER DNA was verified by sequencing, excised from pcDNA3.1/ODCER at the *Afl*II and *Not*I sites, blunt-ended with T4 DNA polymerase, and cloned in the correct orientation into the blunt-ended *Not*I site of the involucrin expression vector pH3700-pL2 to form pH3700-pL2/ODCER.

Generation of transgenic mice The *involucrin-ODCER* transgene was excised from the pH3700-pL2 cloning vector by digestion with *Kas*I and *Ase*I, purified, and microinjected into fertilized B6C3F2 oocytes using standard protocols. After birth, transgenic mice were confirmed by PCR analysis of toe genomic DNA. The *ODCER* transgene was identified by using specific oligo 1 (5′-CCAGGCAGATACTATGTCGCATCAG) that binds in the ODC coding region and oligo 2 (5′-GGTTCAGCATCCAACAAGGCAC) that recognizes the modified ER. Each founder line was backcrossed to C57Bl/6 mice for at least five generations. To activate ODCER in the skin of adult transgenic mice, 1 mg of 4OHT dissolved in ethanol (1.0 mg per 0.1 mL) was topically applied each day to a shaved area of the dorsal skin. Genetically matched wild-type littermates were treated in the same way as controls in all experiments. Double transgenic ODCER/Ras mice were derived by breeding ODCER transgenic mice with Tg.AC transgenic mice possessing the v-Ha-*ras* transgene (Leder *et al*, 1990; Hansen *et al*, 1996). Two hours before sacrifice, all mice were injected i.p. with BrdU (Sigma Chemical, St Louis, Missouri) at a dose of 100 μg per g body weight. The animal protocol for this study was approved by Institutional Animal Care and Use Committee of the Lankenau Institute for Medical Research in accordance with the current US Department of Agriculture, Department of Health and Human Service regulations and standards.

Preparation of skin and tumor homogenates Skin and tumor tissues from transgenic and normal littermates were frozen in liquid nitrogen, ground to a fine powder, and stored at −80°C for subsequent protein and polyamine analyses. In some cases, the epidermis and dermis were separated by plunging excised skin into 55°C water for 20 s followed by scraping. For ODC enzyme activity assays, the epidermal, dermal, and tumor tissues were homogenized in 25 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol, 0.1 mM EDTA, and protease inhibitors including 1 μg per mL each of aprotinin, leupeptin, pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 mM pefabloc. Homogenates were assayed for ODC enzyme activity by quantifying the production of $^{14}CO_2$ from L-[^{14}C]ornithine (New England Nuclear, Boston, Massachusetts) as previously described (O'Brien *et al*, 1975). For polyamine analyses, ground tissues were homogenized in 0.2 N perchloric acid and incubated at 4°C overnight. Dansylated polyamines were separated on a reversed-phase C18 HPLC column (Koza *et al*, 1991). Polyamine values were normalized for the amount of DNA in the tissue extracts.

Primary cultures of epidermal cells Primary cultures of epidermal cells were isolated from 3- to 4-d-old ODCER transgenic newborn pups and their normal littermates by a trypsin flotation procedure (Yuspa and Harris, 1974; Hennings *et al*, 1980). ODCER transgenic pups were distinguished from their normal littermates by PCR genotyping for the ODCER transgene. Cells were plated at 3.0×10^6 cells per 60 mm dish or onto glass coverslips in low-calcium keratinocyte media (EMEM w/o calcium, BioWhittaker Walkersville, Maryland) supplemented with 8% chelex-treated fetal bovine serum and 0.05 mM calcium) and grown at 35°C with 5% CO_2. To induce terminal differentiation, the calcium concentration was raised to 0.14 mM calcium, and the cells were harvested 24 h later. To induce ODC enzyme activity in the ODCER transgenic keratinocytes, cells were treated with 1.0 μM 4OHT for 24–48 h.

Immunoblot analyses Tissue or keratinocytes were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% (w/v) deoxycholic acid, 1% (w/v) NP-40, 1 mM EDTA, containing 1 μg per mL each of aprotinin, leupeptin, pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 mM pefabloc), and passed through a syringe needle multiple times after a 45 min incubation on ice. Lysates were clarified by centrifugation at 15,300 × *g*. for 10 min, and the protein content was determined using the Bio-Rad D/C protein assay kit (Bio-Rad Laboratories, Hercules, California). Protein was separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Massachusetts), and briefly stained with Ponceau S (Sigma Chemical) to verify efficient transfer. Immunoblots were incubated for 1 h in blocking solution (phosphate-buffered saline (PBS) with 10% non-fat dry milk and 0.05% Tween 20) followed by a 2 h incubation with the primary antibody diluted in PBS with 0.1% milk and 0.05% Tween 20. Blots were probed with a rabbit polyclonal K1, K10, K6, loricrin, or involucrin antibody (Covance, Richmond, California), a monoclonal anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, California), a rabbit polyclonal anti-ER antibody (NeoMarkers, Fremon, California), or a rabbit polyclonal anti-IL-1β antibody (R&D Systems, Minneapolis, Minnesota). Filters were reprobed with antibodies against β-actin or tubulin (Sigma Chemical) to verify equal loading of protein. Bound antibodies

were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, New Jersey).

Immunohistochemistry/immunofluorescence Tissues were fixed in 4% *p*-formaldehyde in PBS overnight and embedded in paraffin. Sections of all tissues were stained with hematoxylin & eosin for histopathological evaluation. Sections were deparaffinized, hydrated, and incubated with primary antibody. Primary antibodies used included a rabbit polyclonal antibody against ER (NeoMarkers), rabbit polyclonal antibody against K6 (Covance), a rabbit polyclonal antibody against tenascin-C (Chemicon, Temecula, California), a monoclonal antibody against α-SMA (Biomeda, Foster City, California), and a rabbit antibody against ODC (a kind gift from Oili Heitala, University of Oulu, Finland). Slides were then incubated with the appropriate biotinylated secondary antibody and then with an avidin and biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, California). Immunoreactive cells were localized by incubating the sections with a chromagen solution containing diaminobenzidine and peroxide and then counterstaining with hematoxylin.

BrdU incorporated in cells undergoing DNA synthesis was detected in tissue sections using a kit from Zymed Laboratories (San Francisco, California). Deparaffinized sections were pretreated with 0.1% trypsin for 10 min, blocked with rabbit serum, and then incubated with a rat monoclonal anti-BrdU antibody (Zymed Laboratories) for 2 h at room temperature. The sections were then incubated with a biotinylated goat anti-mouse antibody (Zymed Laboratories) followed by an avidin–biotin–peroxidase complex, revealed in the presence of diaminobenzidine and then counterstained with Mayer's hematoxylin. The number of BrdU-positive nuclei in the basal layer of the epidermis was counted in a total number of at least 1000 basal cells per tissue section under × 200 magnification. The proliferative index was determined by multiplying the number of BrdU-positive cells/total number of counted basal epidermal cells by 100. Sections were also examined for apoptosis using the terminal deoxynucleotidyl transferase-mediated nick end labeling assay according to the instructions of the manufacturer (Apoptag Apoptosis Detection Systems, Serologicals Corp., Norcross Georgia).

For the detection of α-SMA-reactive pericytes and open (functional) endothelial cells in the same histological sections, immunofluorescence staining was performed on paraffin-embedded sections of skin obtained from mice injected i.v. with FITC-lectin (binds to endothelial cells) just prior to sacrifice. Paraffin-embedded sections were deparaffinized, steamed in 0.1 mM sodium citrate, pH 6.0 for 10 min, and incubated with a monoclonal antibody specific for α-SMA (Biomeda) followed by incubation with secondary antibody conjugated to Cy3 (Jackson ImmunoResearch Labs, West Grove, Pennsylvania). Sections were covered with coverslips mounted with Vectashield mounting medium containing DAPI (Vector Laboratories) to counterstain DNA and to visualize the nuclei of all cells. Immunostained sections were examined by fluorescence microscopy using a Zeiss Axiovert 220M microscope (Carl Zeiss, Inc, Thornwood, New York) powered by Axiovision 4.0 software with multi-channel, Z-stack acquisition and 3D deconvolution and analyzed for red (α-SMA) and green (FITC-lectin).

ELISA IL-1β levels of tissue lysates from ODCER2 transgenic mice and their normal littermates were measured. IL-1β protein levels in RIPA skin lysates were assayed using a mouse IL-1β-specific ELISA (Quantikine; R&D Systems) normalized to total protein content measured by the Bradford assay (Bio-Rad Laboratories).

RT-PCR analysis Total RNA was isolated from the skin of normal and transgenic mice using TriReagent (Molecular Research Center, Cincinnati, Ohio). Two micrograms of total RNA was reverse-transcribed using Super Script II RNase H reverse transcriptase (Life Technologies, Gaithersburg, Maryland) and random hexamer primer (Amersham Pharmacia Biotech) at 42°C for 1 h for cDNA synthesis. The reverse transcription product was diluted 15-fold in water, and 10 μL was used as a template for PCR amplification. PCR was performed under standard conditions in a 50 μL reaction mix containing Red Pol polymerase (Gene Choice, Frederick, Maryland), nucleotides, and primers for either K6α or K6β. A constitutively expressed gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used as an internal control, generating a 600 bp PCR product. Briefly, the reactions comprised of 2 min of an initial denaturation step (95°C) followed by 30 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 1 min) followed by a final elongation step of 5 min at 72°C. Twenty microliters of PCR products were electrophoresed on 2% agarose gels. The specific primer pairs used were:

For GAPDH:
forward primer, 5′-TGCTGAGTATGTCGTGGAGTC-3′
and reverse primer, 5′-AGTGGGAGTTGCTGTTGAAGT-3′
For K6α:
forward primer, 5′-AAATACACCACCACCTCCTCCAGC-3′
and reverse primer, 5′-AGCCTGCCACCCAAATACAAC-3′
For K6β:
forward primer, 5′-CACCATCAAATACACCACCAGCG-3′
and reverse primer, 5′-AAGCAGCCAAAAAGAGAAGCGAG-3′

Quantitation of dermal vascularization The skin vasculature was visualized by fluorescent angiography using FITC-labeled lycopersicon esculentum lectin (Vector Laboratories) injected i.v. (100 μg in 100 μL of PBS) via the tail vein of mice 5 min before sacrifice (Inoue *et al*, 2002). Skin tissues were fixed in 4% *p*-formaldehyde and paraffin embedded. Sections were deparaffinized and hydrated, and then cover slips were mounted on slides with Vectastain containing DAPI, sealed, and examined using a Zeiss microscope. All tissues and sections were processed under subdued light. Images were recorded using an Axiocam digital camera, and images of representative views from each section as well as an area away from the tissue to determine background fluorescence were taken using a × 10 objective. To analyze each image for fluorescence intensity, a region in the dermal tissue was circumscribed that excluded the epidermis and hair follicles because of autofluorescence of hair. The mean value of fluorescent intensity of five highlighted areas of each section was calculated as the vessel density for each section using a Macro written for the KS300 imaging software (Zeiss).

Statistical analyses Data are expressed as mean ± SD. Comparison of two groups was carried out with an unpaired, two-tailed Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

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